Kidney International, Vol. 12 (1977), pp. 59–65

Relationship between dialyzer reuse and the presence of anti-N-like antibodies in chronic hemodialysis patients

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Relationship between dialyzer reuse and anti-N-like antibodies in chronic hemodialysis patients. One hundred eleven chronic hemodialysis patients from five dialysis units were tested for the presence of antibodies reactive with red blood cell N substance; 77 patients were available for follow-up study after 18 to 24 months. Initially, 18 patients (16%) had serum anti-N-like antibodies. Thirteen of these patients were in a home dialysis program and were reusing hollow fiber dialyzers. The other five had practiced hollow fiber dialyzer reuse in the past. None of 37 patients using coil dialyzers had anti-N-like antibody. On follow-up testing, anti-N-like antibody persisted in all patients restudied except for one who had a successful renal transplant. Anti-N-like antibodies developed in four additional patients: three were reusing hollow fiber dialyzers at the time, but one had not reused dialyzers for 24 months. Statistical analyses indicated that dialyzer reuse, hollow fiber dialyzers, and home dialysis were significantly related to the presence of anti-N-like antibodies. We interpret the clinical and statistical data to indicate that dialyzer reuse is the major clinical factor in the development of anti-N-like antibody. The likely mechanism involves the prolonged exposure of red cells trapped in the dialyzer to formaldehyde used in preparing dialyzers for reuse. No adverse clinical effects of anti-N-like antibodies were evident in our patients, but hemolysis and acute transplant failure have been reported by others.

Relation entre le réemploi des dialyseurs et la présence d'anticorps anti-N chez les malades en hémodialyse chronique. Cent onze malades soumis à l'hémodialyse chronique dans cinq unités de dialyse ont été étudiés pour déceler la présence d'anticorps réagissant avec la substance N des globules rouges; 77 malades ont pu être suivis ultérieurement pendant 18 à 24 mois. Au début, 18 malades (16%) avaient une substance sérique anti-N. Treize de ces malades étaient en dialyse à domicile et réutilisaient des dialyseurs à fibres creuses. Les cinq autres avaient réemployé des dialyseurs à fibres creuses auparavant. Aucun des 37 malades qui utilisaient des dialyseurs à bobine n'avaient d'anti-N. Au cours de la surveillance l'anti-N a persisté chez tous les malades suivis sauf chez l'un d'eux qui a eu une transplantation rénale réussie. L'anti-N est apparu chez quatre autres malades. Trois de ceux-ci réutilisaient des dialyseurs à fibres creuses à ce moment là mais le quatrième n'avait pas réutilisé de dialyseur depuis 24 mois. L'analyse statistique a montré que le réemploi de dialyseurs, les dialyseurs à fibres creuses et l'hémodialyse à domicile sont liés significativement à la présence de l'anti-N. Le mécanisme vraisemblable implique l'exposition prolongée des globules rouges sequestrés dans le dialyseur à la formaldéhyde utilisée en vue de réemploi. Aucun effet clinique de l'anti-N n'a été mis en évidence chez ces malades mais des hémodialyses et des échecs immédiats de transplantation ont été rapportés par d'autres.

Received for publication November 15, 1976;
and in revised form January 25, 1977.

In the process of hemodialysis, a patient's blood is removed from the usually friendly environment of the vasculature and exposed to a variety of foreign surfaces and substances. In the ideal situation, the blood should return to the patient with the plasma free from added toxic material and with the cellular elements unaltered. Unfortunately, the ideal has not been achieved as evidenced by recent reports of systemic and hematologic toxicity related to hemodialysis [1, 2].

This study was undertaken to explore another apparently dialysis-related phenomenon, the presence of antibodies to red blood cell N-antigen in chronic hemodialysis patients. The reported increased frequency of this generally rare antibody in dialysis patients [3] prompted longitudinal investigation of patients from five dialysis units in this community for the presence of anti-N-like antibodies.

Methods

Patient population. One hundred and eleven chronic hemodialysis patients from five dialysis units were studied initially in the fall of 1974. Seventy-seven of these patients were restudied 18 to 24 months after initial testing. Thirty-four patients had moved to another geographical area or had expired and, thus, were not available for retesting. The characteristics of the dialysis units and patient populations are presented in Table 1. All patients were dialyzed at least three times a week.

Dialysis units A and B used coil dialyzers (Travenol Laboratories, Inc., Morton Grove, IL.) exclusively with no dialyzer reuse. Dialysis unit C had used Kiil dialyzers with reuse until 1970 to 1972 when there was a gradual switch to hollow fiber artificial kidneys (Cordis Dow, Cordis Corporation, Miami, FL). Hol-

Table 1. Characteristics of the patient population at the time of initial study

Unit	Program	Dialyzer	Reuse	No. of patients	Average age (range), *yrs*	Sex[a]	Dialysis duration (range), *months*
A	Center	Coil	Never	28	50 (29 to 63)	16M, 12F	15 (13 to 66)
B	Center	Coil	Never	9	50 (39 to 61)	3M, 6F	28 (12 to 44)
C	Center	HFAK[b]	Previous	20	52 (25 to 65)	20M, 0F	32 (3 to 87)
D	Center	HFAK	Previous	30	49 (22 to 76)	19M, 11F	16 (1 to 42)
E	Home	HFAK	Current	24	49 (25 to 60)	23M, 1F	32 (7 to 71)

[a] M = male; F = female.
[b] HFAK = hollow fiber artificial kidney.

low fiber kidneys were reused for all but a few patients until June, 1973, and again for a six-month period ending five months prior to study. Unit D used Cordis Dow hollow fiber kidneys and reused dialyzers for a ten-month period ending 16 months prior to study. All unit E patients were reusing hollow fiber kidneys at the time of initial study, but stopped reuse 3 to 22 months prior to final study.

Hollow fiber artificial kidneys were delivered packed with 2.5% formaldehyde for sterilization. Kiil dialyzers were routinely sterilized with 2% formaldehyde prior to use. Coil dialyzers were sterilized with ethylene oxide and were not exposed to formaldehyde.

Dialysis units A and C used central delivery systems for dialysate which were sterilized one to three times a week with a dilute formaldehyde solution. Units D and E used individual delivery systems. These systems were sterilized with dilute formaldehyde once a week by home dialysis patients (unit E) and after each dialysis in unit D. Unit B used no formaldehyde in sterilizing its batch delivery systems. All units used water purified by reverse osmosis.

Dialysis units C, D, and E followed the same protocol in preparing dialyzers for reuse. Following dialysis, the blood compartment was flushed with 5 to 20 liters of tap water and then filled with 1.5% formaldehyde. The dialysate compartment was also filled with formaldehyde. Prior to the next dialysis (about 40 hr later), the blood compartment was flushed with 900 ml of heparinized normal saline. The blood line effluent was then tested for the presence of reducing substances with Clinitest tablets (Ames Company, Division Miles Laboratories, Inc., Elkhart, IN). The dialyzer was used only if a negative Clinitest was obtained. The dialysate compartment was flushed with a minimum of 15 liters of sterile dialysate.

Laboratory methods. Venous blood was obtained prior to dialysis, usually 42 hr after the completion of the previous dialysis. Fresh serum was screened for irregular blood group antibodies with commerically available red cells possessing all common antigens (Ortho Selectogen, Ortho Diagnostics, Raritan, NJ) in saline at 4°, 20°, and 37°C. This was followed by indirect Coombs testing with rabbit anti-human serum (Ortho Diagnostics). Serum producing agglutination (0.08 ml) was then incubated with 0.04 ml of red cells possessing specific common antigens (Ortho Identogen) in saline for 30 min at 4°, 20°, and 37°C. Following centrifugation at 3400 rpm, the degree of agglutination was graded from 0 to 4+ using macroscopic and microscopic reading if necessary. Serum producing a pattern of agglutination consistent with the presence of an anti-N antibody was titrated against NN red cells at 20°C. The thermal range of activity of the antibody was determined by incubating serum with NN cells for 30 min at 37°, 32°, 28°, 25°, 20°, 15°, and 4°C. Agglutination was then graded from 0 to 4+. Centrifugation was omitted from this procedure in order to avoid uncontrolled temperature changes.

Direct antiglobulin testing (direct Coombs' test) was performed at room temperature using rabbit anti-human serum (Ortho Diagnostics). Three specimens with positive direct Coombs' tests were tested with specific rabbit anti-IgG and non-antigamma serum (Ortho Diagnostics). An eluate was prepared from one specimen by suspending washed red cells in saline and incubating at 56°C for 10 min. NN red cells were then incubated with the supernatant eluate at 20°C, centrifuged, and examined for the presence of agglutination. Red cell MN phenotypes were determined for 18 patients whose serum contained antibody consistent with anti-N.

In order to explore the immunglobulin nature of the antibody, two sera were incubated with 0.1M 2-mercaptoethanol using Issitt's IgM denaturation technique [4]. Following this, the sera were incubated with NN cells, and the degree of agglutination was determined.

All estimates of agglutination were made by the

Table 2. Occurrence of anti-N-like antibodies on initial study and after 18 to 24 months

	Initial study		Follow-up study		
Dialysis unit	No. of patients	Patients with anti-N-like	No. of patients	Patients initially with anti-N-like	Patients with anti-N-like on follow-up testing
A	28	0	15	0	0
B	9	0	6	0	0
C	20	2	17	1	2
D	30	3	20	3	3
E	24	13	19	10	13
Total	111	18	77	14	18

same individual. The follow-up studies were performed using the same antibody screening and identification methods described above with incubations performed at 20°C.

Howell and Perkins have indicated that the antibody in question is directed against an antigen similar but not identical to red cell N substance [3]. Therefore, in this paper, "anti-N-like" will refer to the antibody found in hemodialysis patients and "anti-N" to the non-dialysis-related antibody.

Clinical laboratory studies, including hematocrit, hemoglobin, peripheral blood smears, and indirect bilirubin, were routinely obtained at monthly intervals from patients in units C and E (same dialysis regimen). In addition, serum haptoglobin and lactic dehydrogenase (LDH) concentrations and reticulocyte counts were obtained on at least three occasions from all patients with anti-N-like antibodies.

Statistical Methods. Patients with anti-N-like antibodies were compared to patients without the antibody with reference to the following clinical variables: dialyzer type, home or center dialysis, dialyzer reuse, blood transfusions, hepatitis B antigenemia, and antihypertensive and corticosteroid medications. The comparisons were made by using a logit transformation of regression analysis for binomial variables, with the dependent variable being the presence or absence of antibodies [5], and by using Fisher's exact method for the 2×2 table [6]. Parametric data were compared by Student's t test.

Results

Initial study. Eighteen of the 111 patients (16%) had serum anti-N-like antibodies on initial testing (Table 2). Statistical analysis indicated that only hollow fiber dialyzers, home dialysis, and dialyzer reuse were significantly related to the presence of anti-N-like. Table 3 lists the patient distributions and the P value (Fisher's exact method) for these significant variables. Dialyzer reuse significantly improved the fit of the logit model when added to dialyzer type and location of dialysis ($P < 0.025$).

Anti-N-like antibodies were found only in patients using hollow fiber dialyzers (Table 4). All of these patients had reused or were reusing dialyzers. Patients who were reusing dialyzers at the time of study had a significantly greater frequency of anti-N-like (13 of 24) than did patients who had stopped reuse from 5 to 16 months previously (5 of 30; $P < 0.003$). The shortest duration of reuse in a patient with anti-N-like antibody was five months.

Follow-up study. Seventy-seven of the original group of 111 patients were able to be restudied (Tables 2 and 4). The 21 patients using coil dialyzers from units A and B remained free of anti-N-like antibodies. One of the 17 patients from unit C continued to have anti-N-like antibody, but another patient developed this antibody during the 21-month interval between testing. This patient had reused hollow fiber dialyzers for a six-month period which ended five months prior to the initial study. Three patients from unit D retained serum anti-N-like antibodies after 22 months; 17 patients remained free of this antibody. Ten of 17 patients from unit E contin-

Table 3. Initial comparison of patients with and without anti-N-like antibodies with reference to type of dialyzer, center or home dialysis, and dialyzer reuse

Clinical variable	No. of patients	Patients with anti-N-like	P value[a]
Type of dialyzer:			
HFAK[b]	74	18	< 0.002
coil	37	0	
Location			
center	87	5	< 0.0005
home	24	13	
Reuse			
none	57	0	< 0.0005
any	54	18	

[a] P value as determined by Fisher's exact method.
[b] HFAK = hollow fiber artificial kidney.

Table 4. Comparison of dialyzer reuse with presence of anti-N-like antibodies

		Initial testing		Follow-up testing		
Dialyzer	Reuse	No. of patients	Patients with anti-N-like	No. of patients	Patients with anti-N-like	Total patients with anti-N-like[a]
Coil	Never	37	0	21	0	0
HFAK	Never	20	0	14	0	0
HFAK	Previous	30	5	23	5	6
HFAK	Current	24	13	19	13	16
Total		111	18	77	18	22

[a] Includes those patients who were positive on initial testing but did not complete the study.

ued to have anti-N-like antibodies, and three additional patients developed antibodies during the interval after initial testing but prior to discontinuation of reuse. Thus, 18 of the 77 patients (23%) had serum anti-N-like antibodies on completion of the study. Thirteen of the 14 patients with serum anti-N-like antibodies on initial testing continued to have anti-N-like antibodies after 18 to 24 months. One patient received a related donor kidney and attained good renal function 11 months prior to repeat testing. Anti-N-like antibodies were detectable at seven months but not at 11 months. Six patients from unit E continued to be free of anti-N-like antibodies; 13 patients, however, continued to have antibodies despite discontinuing dialyzer reuse 7 to 19 months prior to final testing.

Clinical and laboratory findings. None of the patients with serum anti-N-like antibodies had clinical or laboratory evidence of hemolysis on initial or follow-up study. The hematocrit values from patients with and without antibodies from units C and E were compared at initial and final study. On initial testing, the average hematocrit values for patients with serum anti-N-like antibodies was 29 ± 9% (SD); for patients without antibodies, it was 30 ± 8%. After 18 to 24 months, the averages were 31 ± 5% and 33 ± 8%, respectively. The averages were not statistically different on either occasion. Two patients with serum anti-N-like antibodies required blood transfusions during the study, both following major abdominal surgery.

One patient with anti-N-like antibody received a related donor kidney transplant without problems. Another patient received two cadaver kidneys with rejection occurring at seven days and five months. Both kidneys were flushed with warm saline immediately prior to completion of the vascular anastamoses and functioned well initially.

MN phenotypes were determined in 18 patients with anti-N-like antibodies. Eleven were MN, six were MM, and one was NN. Titers of anti-N-like antibodies were determined for 13 sera and were generally very low: six were 1:1, three 1:2, and one each was 1:4, 1:8, 1:16, and 1:32.

The variation in thermal activity was great; however, all anti-N-like antibodies produced agglutination at 20°C. Three were active at 37°C, yet these patients did not have signs of ongoing hemagglutination or hemolysis. Two of these patients possessed an MM phenotype. The third patient was unique in that his anti-N-like antibody was active at 37°C against the NN cells used to identify the presence of the antibody but not against his own NN cells at temperatures above 28°C.

Direct Coombs' tests were positive with 5 of the 15 samples tested. Specific anti-IgG serum testing was positive with one and negative with two samples. The latter two samples yielded positive tests with anti-nongamma serum. Four of the patients with positive direct Coombs' tests had MN red cell phenotypes; one had type MM. The eluate obtained from the red cells which had given a positive anti-IgG test did not produce agglutination of NN cells. The sera from three of the five patients with positive direct Coombs' test also contained nonspecific cold agglutinins. Serum incubated with 2-mercaptoethanol no longer produced agglutination of NN red cells.

Discussion

Anti-N is an antibody directed against N antigen of the red cell MN system. Removal of one N-acetylneuraminic acid group (NANA) from MM antigen produces NN antigenicity. Removal of another NANA inactivates NN antigen. Thus, the MN system appears to consist of a structural precursor (T antigen), with one added NANA yielding NN antigen and a second NANA producing MM antigen [7]. Anti-N is a rarely found cold agglutinin, which even more rarely causes hemolysis [8, 9].

This study confirms the increased frequency of anti-N-like antibodies in patients on chronic hemodialysis. Howell and Perkins initially described this

phenomenon in 12 of 416 hemodialysis patients screened for transplantation [3], and McLeish, Brathwaite, and Peterson subsequently noted it in six of 40 patients [10]. Recently, Harrison et al detected anti-N-like antibodies in 15 of 33 home hemodialysis patients who were reusing dialyzers and in none of 28 center patients who had never reused dialyzers [11].

Twenty-two patients from an initial group of 111 (20%) were found to have anti-N-like antibodies during this study. Analysis of the initial testing data indicated that three clinical variables–home dialysis, hollow fiber dialyzers, and dialyzer reuse–were significantly related to the presence of anti-N-like antibodies. Home dialysis *per se* is unlikely to be biologically significant in the development of anti-N-like antibodies since home patients are drawn from the same population, use identical dialysis equipment and water purification, follow the same treatment schedule, and are trained in the same techniques as used for center dialysis patients.

In this study, all patients with anti-N-like antibodies used hollow fiber dialyzers; however, 9 of 12 patients in Howell and Perkins' series [3] and 2 of 15 in Harrison's study [11] used Kiil dialyzers. On initial study, none of the 20 patients dialyzed with hollow fiber kidneys without reuse had anti-N-like antibody, in contrast to 18 of 54 patients who reused hollow fiber kidneys and had anti-N-like antibodies ($P < 0.005$). The patients who did not reuse dialyzers had been on dialysis for an average duration of 24 months shorter than patients who reused dialyzers. None of 14 patients who used hollow fiber dialyzers without reuse, however, developed anti-N-like antibody during the subsequent 22 months. None of the patients using coil dialyzers had anti-N-like antibody on initial or follow-up study (see Table 4). While three patients from unit E developed anti-N-like antibodies during the study when still reusing dialyzers, an exception is the patient from unit C who also developed anti-N-like antibody but had stopped dialyzer reuse five months prior to initial study and had not reused dialyzers during the 21 month study interval. He is the only patient in this series definitely known to have developed anti-N-like antibody at a time when dialyzers were not being reused. Ongoing or previous dialyzer reuse significantly improved the fit of the regression analysis when added to the other two statistically significant variables, location and dialyzer type. We conclude that dialyzer reuse is the most significant clinical variable related to the development of anti-N-like antibodies in hemodialysis patients. The development of anti-N-like antibody, however, in a patient who had not reused dialyzers for 26 months suggests that single use of formaldehyde-sterilized hollow fiber artificial kidneys may on occasion be sufficient to generate anti-N-like antibody.

The mechanism responsible for the development of anti-N-like antibody appears to involve formaldehyde, as suggested by Howell and Perkins [3]. We have recently demonstrated that MM red cells are altered by exposure to dilute formaldehyde, such that they agglutinate on incubation with anti-N-like antibody [12]. Formaldehyde is known to the capable of altering protein structure [13] and presumably antigenicity. The mechanism by which formaldehyde alters red cell MN antigenicity must account for the development of anti-N-like antibodies in patients with MM, MN, and NN phenotypes. No *in vitro* data is available to detail this mechanism, but we feel that a direct chemical reaction between formaldehyde and a portion of the red cell MN structure is responsible for the generation of a foreign antigen which stimulates anti-N-like antibody formation. Formaldehyde could form a methylene glycol ester [13] with the NANA groups of M and N substance, thus masking their presence, or its reducing action could indirectly induce purely conformational changes. Formaldehyde exposure in patients on hemodialysis has been recently reported to alter red cell metabolism by NAD reduction, thus causing hemolytic anemia [14]. This mechanism seems unlikely in this setting.

We have observed agglutinated red blood cells and fragments adherent to the fibers of dialyzers prepared as if for reuse, and others have found all cellular elements on the membranes of dialyzers following a single use [15, 16]. Thus, reuse sterilization provides the opportunity for lengthy exposure of red cell antigenic surfaces to formaldehyde during the 40 hr of sterilization prior to the next dialysis. Subsequent hemodialysis may then wash some of the altered antigens into the circulating blood for delivery into the patient. If these substances are sufficiently antigenic and the patient's immune system competent, anti-N-like antibody will be formed.

Anti-N-like antibody has been reported to disappear within 7 to 11 months following successful renal transplantation [3]. In the one successfully transplanted patient in our series, anti-N-like antibody was still detectable after seven months, but had disappeared at 11 months. The anti-N-like antibodies have not disappeared in any of the patients remaining on dialysis, despite the discontinuation of dialyzer reuse 9 to 38 months prior to the end of the study (average, 18 ± 11 months). The persistence of anti-N-like antibodies could be related to continued generation of a very small amount of altered N antigen due to the residual formaldehyde present after

even the most vigorous dialyzer preparation. The Clinitest method for detecting residual formaldehyde is relatively crude and will only detect concentrations above 15 μg/ml. The method used to prepare new hollow fiber dialyzers for use in units C, D, and E could leave a residual formaldehyde concentration approaching that limit [17]. This residual formaldehyde effect on red cells does not seem sufficient to generate the development of detectable anti-N-like antibody in most individuals, but it may well be able to generate enough antigen to continuously stimulate previously sensitized anti-N-like-producing cells. On occasion, this residual formaldehyde may produce a quantity of altered N-antigen sufficient to generate anti-N-like antibody in a patient who has never reused dialyzers or who has discontinued dialyzer reuse, as in the patient from unit C described previously.

The presence of anti-N-like in dialysis patients is usually not manifested clinically. A patient reported by Harrison et al had increased transfusion needs until dialyzer blood lines were insulated and the dialysate temperature raised to 38°C [11]. In our series, there was no evidence of hemolysis, increased transfusion needs, or anemia disproportionate to that seen in patients without anti-N-like antibodies. The direct Coombs' test was positive in 5 of 15 patients with anti-N-like antibodies, in contrast to its presence in all of the patients reported by McLeish et al [10]. None of the 12 patients reported by Howell and Perkins had a positive Coombs' test [3]. The inactivation of the anti-N-like antibody on incubation in 2-mercaptoethanol suggests that it is of the IgM class [4].

Since anti-N-like antibodies are reactive at cold temperatures, any MN or NN phenotype patient possessing anti-N-like antibody who receives a chilled organ in transplant has the potential for autoagglutination of red cells in the cold graft organ. Two instances of acute graft failure due to this phenomenon have been reported [3, 18]. Since MN group antigens are found in mammary glands, salivary glands, kidney, and liver [19], antibodies to these tissues may produce deleterious effects not apparent at this time.

The practice of reusing dialyzers has important economic considerations. Problems with altered membrane characteristics and pyrogen reactions do occur, but have not been major in our experience. Anti-N-like antibodies have not caused apparent clinical problems in our patients. The generation of an antibody directed against a substance present in many tissues, however, is significant enough in itself to warrant consideration of discontinuance of dialyzer reuse or the adoption of a non-formaldehyde process for resterilizing dialyzers and perhaps even for initial dialyzer sterilization.

Achnowledgments

This work was presented in part at the Eighth Annual Meeting of the American Society of Nephrology, Washington, D. C., November 25–26, 1975. We acknowledge the technical assistance of Vivian Mega, M. T. (ASCP) BB; the cooperation of Drs. Richard Holman, Wagner Schorr, Michael Persoff, Jeffrey Jennings, S. Robert Contiguglia, Melvyn Klein, and Jeffrey Mishell in providing access to their patients; the assistance of Drs. Philip Archer and Richard Jones in the statistical analyses; and the assistance of Daisy Rodarte in preparing the manuscript.

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